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THE MANUFACTURE AND STUDY OF HEMOGLOBIN - SALINE SOLUTION.(U)
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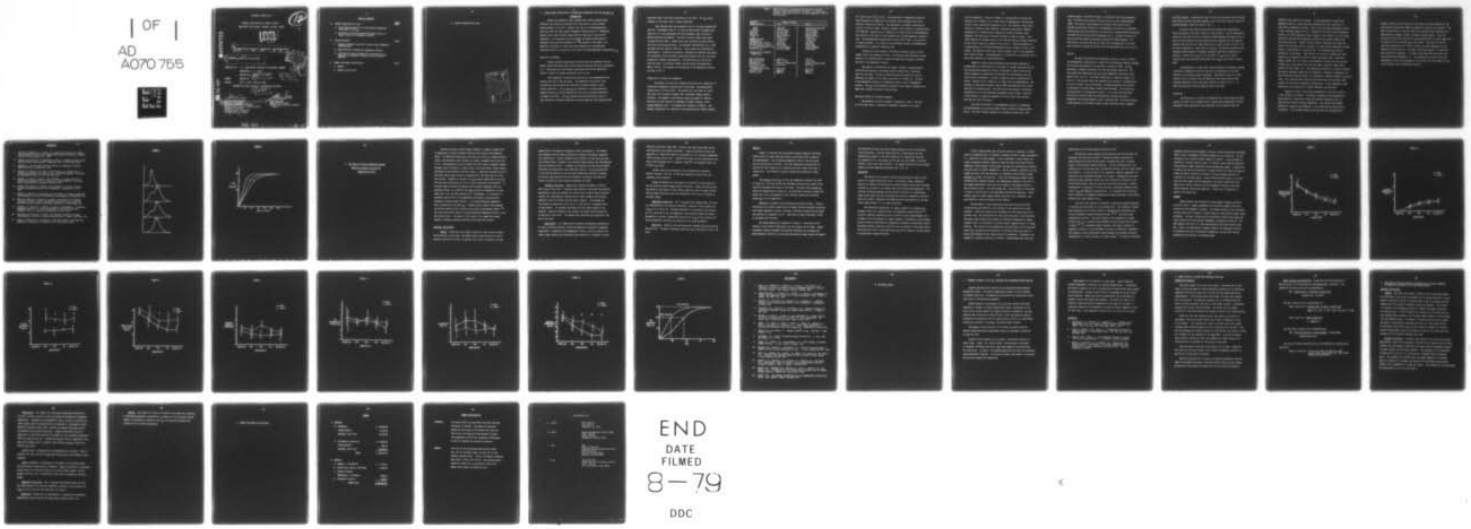

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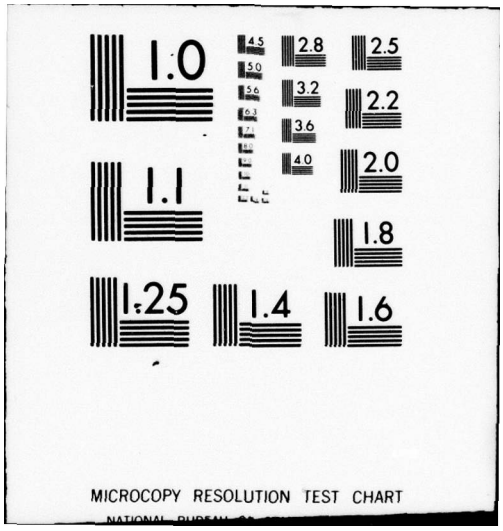
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A PROPOSAL SUBMITTED BY

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1979

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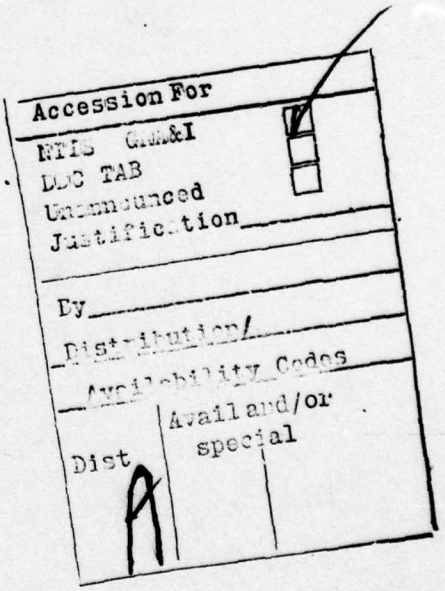

1. LARGE VOLUME PREPARATION OF PYRIDOXALATED HEMOGLOBIN WITH HIGH *IN VIVO* P_{50}

INTRODUCTION

Stroma free hemoglobin (SFH) prepared from freshly outdated human blood has very attractive characteristics that make it a most suitable plasma expander (1,2,3,4,5). However, the loss of 2,3 diphosphoglyceric acid (2,3, DPG) and other organic phosphate ligands results in a hemoglobin solution with a much stronger oxygen affinity than that of the red cell hemoglobin. This advantage can be corrected by chemical modification of hemoglobin with pyridoxal phosphate (6). The purpose of this paper is to describe a technique for simple and rapid preparation of pyridoxalated hemoglobin (SFH-P) in volumes up to 20 liters at 20 gm% hemoglobin concentration.

Materials and Methods

Freshly outdated packed human red blood cells were obtained from the Michael Reese Blood Bank Center and the American National Red Cross, Washington, D. C. The packed cells were washed three times with sterile physiologic saline to remove all plasma contaminants prior to use.

Total hemoglobin concentrations and the per cent methemoglobin were obtained with the 1L 282 cooximeter. The hemoglobin dissociation curve was obtained from the Hem-O-Scan (American Instrument Company, Silver Springs, Maryland). The *in vivo* P_{50} was determined on plasma hemoglobin samples obtained at various hematocrits during total exchange transfusion of baboons with SFH-P. The P_{50} obtained from the dissociation curve is then corrected to standard conditions of pH and pCO_2 (ph-7.40, pCO_2-40 mm Hg)

using the "Bohr" coefficient determined for our SFH-P. All P_{50} values reported in this paper refer to these conditions.

Total phospholipids were assessed by use of the Hycel phospholipid test kit. Prothrombin time (P. T.) and activated partial thromboplastin time (A.P.T.T.) were determined with the Fibrometer (BBL, Division of Becton Dickinson and Company). The Citrol controls (Dade) were reconstituted with the SFH-P. Normal values were obtained by reconstituting the Citrol with deionized water. The enzymatic determination of 2, 3 DPG was made with the sigma No. 35-UV kit. Total protein was determined by refractometry. Crystalline pyridoxal-5'-phosphate was obtained from Sigma. All components of the filtration system were obtained from the Millipore Corporation, Bedford, Massachusetts. Ultrafiltration was carried out with the C-Dak 1.3 artificial kidney from the Cordis Dow Corporation, Miami, Florida. All preparation and handling of the hemoglobin solution was done at 5°C. P.

Preparation of Stroma Free Hemoglobin

The washed red blood cell suspension typically has a hematocrit of 75-80% and a hemoglobin concentration of 23-25 gm%. The methemoglobin concentration is 0-1% of total. The packed cells are lysed in a stainless steel cell disruption chamber (Parr Instrument Company, Moline, Illinois). The chamber is equilibrated under N_2 pressure of 1000 psi. The cells are then released to atmospheric pressure through a valve causing immediate lysis. The chamber has a capacity of 1200 ml. The stromal lipoproteins are removed by acid precipitation of 1200 ml batches

Table 1. Characterization of Pyridoxalated Hemoglobin Solution (SFH-P) without DEAE-Sephadex Treatment. For comparison purposes, characteristics of a stripped hemoglobin solution is also given.

SOLUTION CHARACTERISTICS	RANGE OF VALUES	
	SFH	SFH-P
Yield	100% SFH	68-75% SFH-P Bal-SFH
Sodium	135-145 mEq/L	135-145 mEq/L
Potassium	3.5-4.5 mEq/L	3.5-4.5 mEq/L
Chloride	90-110 mEq/L	90-110 mEq/L
Osmolarity	290-310 mOsm/L	290-310 mOsm/L
ph	7.35-7.45	7.35-7.45
Hemoglobin (Hb)	7.0-8.0 gm%	7.0-8.0 gm%
Methemoglobin	3.0-8.0%	3.0-8.0%
Non-hemoglobin Protein	5.0-10.0%	5.0-10.0%
Colloid oncotic Pressure	20-25 mmHg	20-25 mmHg
2, 3 Diphosphoglyceric acid	1.5-3.0 Mlgm/tb	1.5-3.0 MlgmHb
Plasma Hb P_{50} (normalized to standard conditions)	12.0-14.0 mmHg	20-24 mmHg
Hill Coefficient	1.6-2.5	1.6-2.5
Bohr coefficient	-0.2 - -0.36	-0.2- -0.3
Phospholipids	5-10% of lysate value	5-10% of lysate value
Prothrombin time	12-13 sec	12-13 sec
Activated Partial thromboplastin time	27-31 sec	27-31 sec
Sterility test	Negative	Negative
Toxicity test	Negative	Negative

at a pH of 5.6-5.7 with 1 N Hcl. The precipitate is immediately packed by centrifrugation at 20,000 rpm for 20 minutes using a Beckman JCF-Z rotor that has a capacity of 1300 ml. The supernatant is promptly readjusted to a pH of 7.4 with 1 N NaOh. Glucose is added to the solution in a concentration of 5-100% mg%. Ascorbic acid is added on a 4:1 molar ratio with methemoglobin. The solution at this point in the procedure has a 20 gm% hemoglobin concentration. The methemoglobin concentration is under 10% of total. The solution is stored overnight. As a result of the overnight storage, the methemoglobin concentration is typically reduced by 50%.

Filtration of the solution is carried out through a series of cartridges, under a N_2 pressure of 40 psi. At the end of the filtration, approximately 1L of deionized water is passed through the cartridges to minimize the loss of the hemoglobin solution.

The removal of electrolytes and organic ligands is accomplished by ultrafiltration against a continuous counter current flow of deionized water for two hours. The use of deionized water was found to be just as effective for removal of 2,3, DPG as the use of 0.1 M NaCL (7). Dilution of the hemoglobin solution is prevented by using a 10 psi transmembrane pressure. The P_{50} of the hemoglobin solution at this stage is between 12-14 mmHg under standard conditions of pH and pCO_2.

CO-valent Binding of Pyridoxal Phosphate

The hemoglobin solution (18-20%) is adjusted to a pH of 7.40 with 0.1 M Tris-HCL buffer. Pyridoxal-5'-phosphate is added on a 4:1 molar

ratio to hemoglobin. Glucose is added to a concentration of 100 mg% and ascorbic acid is added on a 4:1 molar ratio to methemoglobin concentration. The pH is again checked and adjusted to 7.40. The solution is then transferred to a stainless steel reservoir, (Millipore Corporation). Complete deoxygenation is accomplished by pumping the solution through a Blood Oxygenator (William Harvey) and using nitrogen in place of oxygen. Overnight bubbling of nitrogen results in the solution having an oxygen saturation of less than 5% and an 0_2 content of less than 1%. Sodium borohydride at a concentration of 0.02 moles/mM hemoglobin/liter) is added in 50 ml of 10^{-3} N NaOH. The addition is made very slowly, through a port in the oxygenator with a 50 ml syringe. The deoxygenation is continued for an additional 10-12 hours after addition of sodium borohydride.

Removal of excess sodium borohydride and pyridoxal phosphate is accomplished by ultrafiltration for two hours in a manner described earlier. The pyridoxylated hemoglobin solution (18-20 gm%) is then diluted to the desired hemoglobin concentration and adjusted for electrolytes using the renal dialysis concentrate. Glucose is added to yield a concentration of 50 mg%. Ascorbic acid is added on a 4:1 molar ratio to methemoglobin. The solution is then passed through a sterile CWSS cartridge and 0.22 m filter (293 mm) into transfer packs. The sterility of the solution is tested by inoculation of 5 ml into anaerobic and aerobic culture broths. Toxicity of the solution is tested by injection of 0.2 ml of the final solution into the tail vein of 4-6 mice.

The yield of the SFH-P in the hemoglobin solution is determined by electrophoresis on cellulose acetate strips in EDTA-Tris-Borate buffer pH 8.0. The SFH-P fraction migrates at a distinctly faster rate. The

relative amount of the SFH-P fraction is determined from the densitometric scan. The SFH-P can be purified from the solution using a DEAE-Sephadex (A50). The column is equilibrated with 0.01M Tris-HCl buffer pH 8.0. The SFH and the SFH-P fractions in the hemoglobin solution can be differentially eluted from the column with a pH and ionic strength gradient consisting of 0.01M Tris pH 8.0 and 0.1M Tris-Maleate pH 5.3. The SFH-P fraction can also be purified readily by batching technique with DEAE-Sephadex. The SFH fraction is eluted from the gel with 0.1M tris-maleate pH 6.9 and the SFH-P fraction is eluted off the gel with the same buffer but at a pH of 6.0.

Results

The data characterising the hemoglobin solution is given in Table 1. The methemoglobin concentration is under 10% and essentially remains unchanged for at least three months if the solution is stored at -20°C. The P_{50} corrected to standard conditions of pH and pCO_2 ranges from 20-24. The Hill coefficient determined from the plasma hemoglobin dissociation curve varies from 1.8 to 2.5. The "Bohr" coefficient is typically between -0.2 and -0.3. The proteins other than hemoglobin are present in concentrations of 0.6 to 0.8 gm%. The colloid oncotic pressure exerted by the solution as determined on plasma samples ranges from 20-25 mmHg. The solution has no procoagulant or anticoagulant activity. It is sterile and non-toxic. The yield of the SFH-P from the pyridoxalation reaction with SFH as determined from the densitometric scan is typically 70%. The SFH-P fraction in the solution can be readily raised to more than 85% by batch treatment

with DEAE Sephadex. Densitometric scans of SFH; SFH-P solution, SFH-P solution after batch treatment with DEAE Sephadex, and of the SFH-P fraction eluted off the DEAE-Sephadex column are shown in Fig. 1.

As can be seen from the scans the batch method does significantly raise the content of the SFH-P fraction in the solution. Typical dissociation curves for SFH, SFH-P and SFH-P treated with DEAE Sephadex are shown in Fig. 2. The pH of the solutions are those obtained after tonometry with the same gas mixtures that are fed to the Hem-O-Scan and are usually close to a pH of 7.40. The pO_2 is adjusted to a value close to the P_{50}. The SFH and the SFH-P curves represent plasma samples from baboons exchange transfused to hematocrits of below 5%. The SFH has a P_{50} of 13.2, the SFH-P, a P_{50} of 22.0, (SFH-P fraction 70%) and the batch purified SFH-P has a P_{50} of 28.0 (SFH-P fraction 85%).

Electrophoresis of plasma SFH-P samples obtained from baboons exchange transfused to hematocrits below 5% show that the percentage of the SFH-P fraction in the solution remains unchanged. Densitometric scan of an SFH-P solution prior to infusion into the baboon and the plasma obtained after exchange transfusion of the same SFH-P solution is shown in Fig. 3. In both cases the SFH-P fraction comprises 70% of the total.

Discussion

In the red cell, a variety of mechanisms that involve organic ligands such as 2,3, DPG, ionic strength and pH, regulate the oxyhemoglobin affinity and permit rapid saturation of the hemoglobin in the lung and ready off-

loading of the oxygen to the tissues. In the preparation of stroma free hemoglobin solution, these regulatory mechanisms are lost, resulting in a high affinity hemoglobin solution. There are two possible physiologic effects of this increased oxygen affinity. Transfusion of a high affinity hemoglobin solution can either impede the flow of oxygen to the tissues or it can trigger some other autoregulatory mechanism to maintain the necessary oxygen flow for tissue viability. Previous studies from our laboratory have shown the latter to be the case; that is, infusion of a high affinity hemoglobin solution results in the decrease in tissue pO_2 (8). This decreased pO_2 could be detrimental to the animal. This has led to a search for ligands that would reduce the oxy-hemoglobin affinity to match the normal state. Additon of organic ligands such as 2,3 DPG to the hemoglobin solution does not maintain a normal P_{50} in the animal due to the very rapid clearance of these small molecules by the kidney. (9). This problem was resolved by co-valent linkage of and organic phosphate ligand such as pyridoxal-5'-phoshpate to hemoglobin (6). This irreversible binding results in a permanently rightward shifted dissociation curve. This technique when originally described dealt with extremely small volumes and non-clinical hemoglobin concentration. It has subsequently been modified to allow preparation at higher hemoglobin concentration.

However, no data on the relative proportion of the SFH-P fraction in the solution is available. The present technique allows preparation of large volumes of irreversibly linked pyridoxalated hemoglobin that can essentially be freed of stripped hemoglobin. The covalent bond between pyridoxal-5'-phosphate and hemoglobin is not broken during intra-vascular circulation. This is demonstrated by the fact that the electrophoretic

pattern of SFH-P prior to infusion in the animal, and that obtained for the same solution from plasma samples at hematocrits below 5% are identical. The SFH-P fraction which is typically 70% by our pyridoxalation technique with DEAE-Sephadex treatment, remains the same in plasma samples. Thus, the mixing of the pure SFH-P and SFH in varying proportions will allow preparation of solutions with any desired P_{50} in the animal. The value for the Hill coefficient obtained for our SFH-P solutions are quite comparable to that reported in the literature. The "Bohr" coefficient however, is lower than that reported for hemoglobin. A similar diminished effect has been noted for pyridoxalated hemoglobin (11). This preparation technique offers the adavantage of large scale preparation of pure FHF-P with low oxygen affinity and no apparent loss of physiologic function.

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FIGURE 1

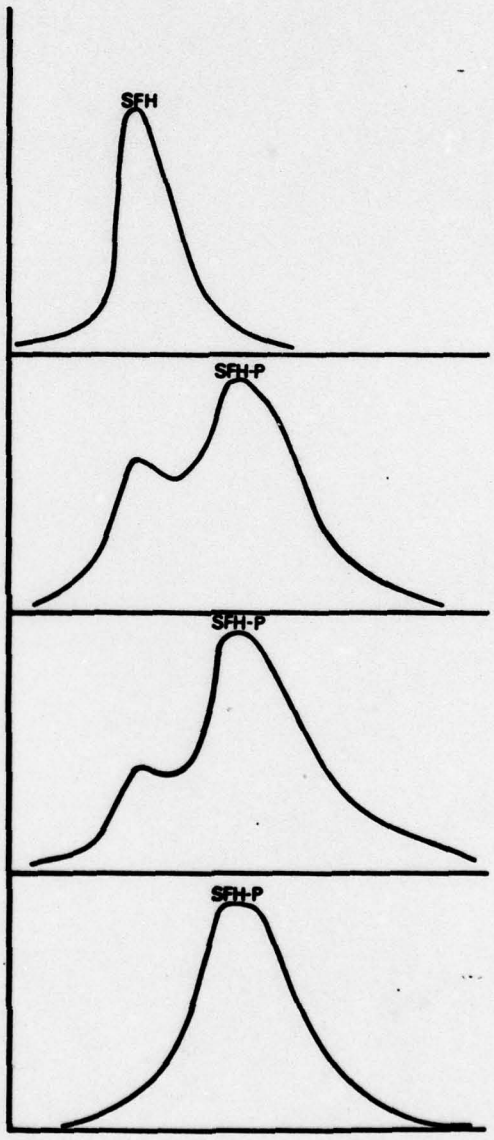
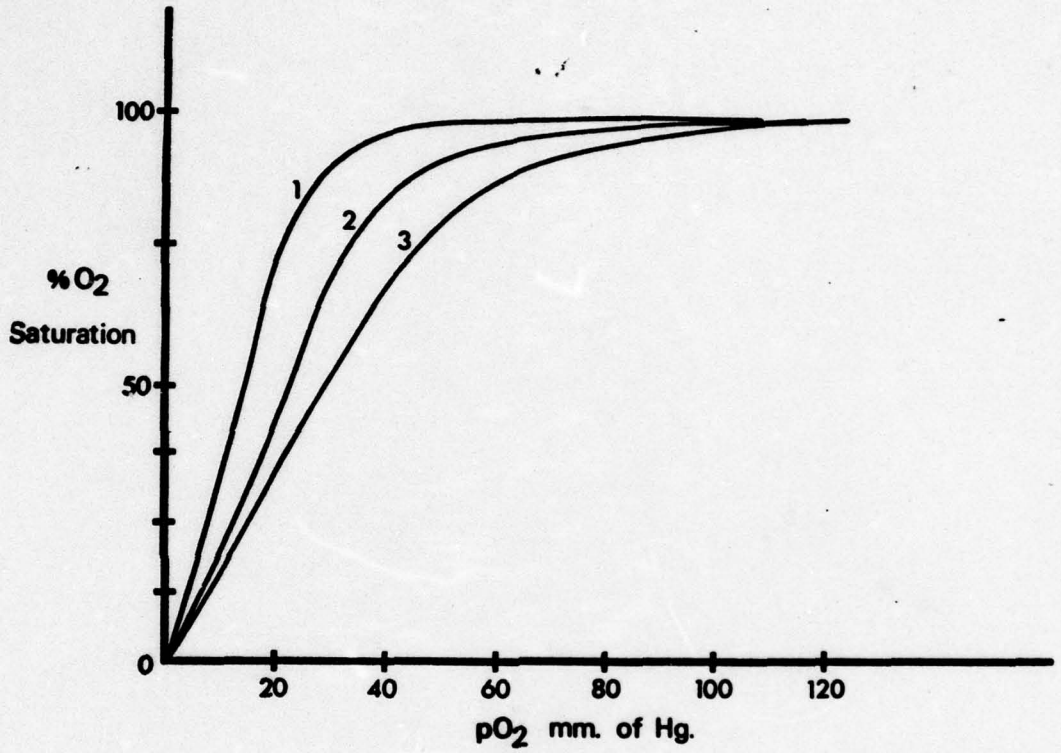


FIGURE 2



2. The Effect of Altered Hemoglobin-Oxygen Affinity on Oxygen Transport by Hemoglobin Solution

We have previously studied oxygen transport in baboons rendered free of erythrocytes by total exchange transfusion with stroma free hemoglobin (SFH).[1] An important consideration with the use of SFH as an oxygen-carrying fluid is the abnormally high affinity for oxygen, presumably due to the loss of 2,3 - diphosphoglyceric acid (2,3-DPG) and other organic phosphate ligands. Although oxygen consumption, cardiac output, and arteriovenous oxygen content difference are maintained at baseline values, a significant decrease occurs in the mixed venous oxygen tension to compensate for the high affinity of the SFH solution. Attempts to increase the P_{50} by merely adding DPG to the solution have been unsuccessful due to its short intravascular persistence.[2] It has been shown by Benesch and Benesch that pyridoxal phosphate (PLP) has analogous effects to 2,3-DPG on the oxygen affinity of hemoglobin, and competes with it for the same binding site.[3] Furthermore, it is possible to covalently bond the PLP to the hemoglobin and produce a solution with a permanently lowered oxygen affinity - a so-called permatized hemoglobin - with a significantly elevated P_{50}. Although several reports have compared the SFH and permatized SFH solutions using various experimental models,[4,5] the actual physiologic benefit of the pyridoxalated hemoglobin (SFH-P) is not yet entirely clear. The object of this report is to compare the oxygen dynamics in baboons exchange transfused with either SFH or SFH-P.

MATERIALS AND METHODS:

Animals. Eight adult male baboons (20-22 kg.) were the test animals. On the morning of each study, the baboons were tranquilized with an intramuscular injection of 150 mg. of ketamine and 0.5 mg. of atropine, and then

sedated with 12-15 mg/kg of thiopental sodium intravenously. The trachea was intubated, and the baboon paralyzed by frequent intravenous injections of d-tubocurarine. Plastic catheters were inserted into the aorta and vena cava through femoral cutdowns, and a balloon-tipped catheter was flow-directed into the pulmonary artery. A catheter was inserted into the urinary bladder. The baboon was mechanically ventilated with room air in the prone position. In the baseline period the rate and tidal volume were adjusted to produce an arterial pCO_2 between 35 and 45 millimeters of mercury, and these ventilator settings were held constant throughout the study.

Exchange Transfusion. Baboons were randomly assigned to an SFH or SFH-P group of four each. Following stabilization of the animal and baseline measurements, blood was withdrawn in 50 milliliter aliquots from an arterial catheter and simultaneously replaced with an equal volume of the selected hemoglobin solution infused into the venous catheter. The exchange was interrupted at hematocrits of 20, 10, 5, 2, and zero for a complete series of measurements. The exchange continued until the lowest hematocrit was achieved. When zero hematocrit was reached, the animal was maintained for an additional three hours. The baboons were sacrificed and autopsied at the end of the study.

Measurements. All samples were collected and separated anaerobically in order to perform analyses of both the plasma and erythrocytic hemoglobin compartments. Hemoglobin and methemoglobin levels, as well as arterial and venous oxygen contents and saturations were measured in a cooximeter (instru-

mentation Laboratory, Model 282). Arterial and venous blood gases and pH were measured using standard electrodes. Oxygen dissociation curves were generated on a Hem-O-Scan (American Instrument Co.) at a constant temperature ($37^{o}C$) and pCO_2 (40 mm. Hg.). Standard corrections for pH, temperature, and pCO_2 yielded in-vitro (pH 7.4, pCO_2=40, temp=$37^{o}C$) and in-vivo (animal conditions) P_{50} values.

Cardiac output was determined by the thermodilution technique. Arterial pressure, CVP, PAP, and PAW were measured from the aortic and pulmonary artery catheters.

Oxygen consumption was calculated as the product of the cardiac output and arterious venous oxygen content difference. Oxygen availability was calculated as the product of the cardiac output and arterial oxygen content, and the oxygen extraction ratio was determined as the ratio of consumed to available oxygen.

Hemoglobin Preparation. SHF is prepared from outdated human red cells by a modification of our previous technique[1], yielding a final product with a P_{50} of 12 to 14 mm. Hg. Pyridoxal phosphate is then added to the solution on a 4:1 molar basis with the hemoglobin, and covalently bound with sodium borohydride to provide a permatized solution with a P_{50} of 20 to 25 mm. Hg.[6] The two hemoglobin solutions are identical in all other parameters.

Statistics. Results in the two groups were compared using the Wilcoxon Rank-sum test. The major differences identified were significant at the P .05 level.

RESULTS:

Figure 1 illustrates the relationship between hematocrit and hemoglobin values for whole blood and plasma in each group after correction for methemoglobin. The circulating hemoglobin values in the two groups are not statistically different. The final hemoglobin concentrations for the SFH and SFH-P groups are 4.3 grams per cent ±0.2 (SEM), and 5.0±).4, respectively. The volumes of solution infused were equivalent in both groups.

The in-vitro plasma P_{50}'s of the two hemoglobin solutions are shown in Figure 2a. The data confirms the different affinities for oxygen of the solutions throughout the course of the study. The whole blood in-vivo P_{50} values are demonstrated in Figure 2b. No significant difference between the group occurs until the hematocrit falls below 20, despite the different plasma P_{50}'s at all hematocrits.

Hemodynamic parameters were maintained with both solutions. Figure 2 illustrates that cardiac output does not change following exchange transfusion with either hemoglobin solution, although a difference between the two groups was observed at a hematocrit of 10. There were no major differences in MAP or HR between SFH and SFH-P.

The oxygen dynamics are presented in Figure 4. There were no differences in the arterial blood gases, and the figures are not shown. Oxygen consumption remains unchanged from baseline throughout the exchange with both hemoglobin solutions, as does the arteriovenous oxygen content difference.

The individual arterial and venous oxygen contents are also not different in the two groups. The mixed venous pO_2 fell in both groups, but was significantly greater in the SFH-P animals at all hematocrits below 20. At a hematocrit of 5, the values are 13.8 mmg. Hg. $\pm$2.4 (SEM), in the SFH solution, and 31.3$\pm$3.0 with the SFH-P. The oxygen extraction ratio increased equally with both hemoglobin solutions from .15 to .47.

DISCUSSION:

The results of this study illustrate the physiologic effect of manipulation of oxygen dissociation curve. As previously shown, hemoglobin solution is capable of maintaining circulatory dynamics during total exchange transfusion, and there is no benefit seen with pyridoxalated hemoglobin. The principle difference between the SFH and SFH-P animals is a significantly higher mixed venous pO_2 with the permatized solution at hematocrits below 20. Since this number is generally considered the best approximation of the mean tissue oxygen tension, it is a major difference.

The traditional determinants of oxygen transport are pulmonary gas exchange, blood flow, hemoglobin mass, and hemoglobin-oxygen affinity.[7,8] A precise regulatory mechanism exists to satisfy tissue oxygen demands, and a change in any one parameter of oxygen transport is balanced by other changes. We have previously determined the compensatory response to normovolemic anemia following exchange transfusion with SFH to be an increase in the oxygen extraction ratio and a fall in the mixed venous pO_2 with no change in cardiac output or arteriovenous oxygen difference.

A fall in mixed venous pO_2 can be the result of a decrease in cardiac output or hemoglobin mass, an increase in oxygen affinity or oxygen consumption, or a combination of these changes. In this experiment, cardiac output and oxygen consumption remained constant at baseline values with SFH and SFH-P/ Hemoglobin values dropped in both groups, but were not significantly different from each other at similar hematocrits. The only variable, therefore, was the oxygen affinity of the two solutions, as indicated by the different plasma <u>in-vitro</u> P_{50} values (12 versus 22). Since the arteriovenous oxygen content difference also remained constant, the compensatory response to the shift in the oxygen dissociation curve had to be a change in the oxygen tension at which unloading occurs - the mixed venous pO_2. The principle is illustrated in Figure 5, and the data in the experiment confirms the hypothesis. SFH-P unloads oxygen at higher oxygen tensions than SFH, and is therefore more physiologically suited to minimize tissue hypoxia.

The difference in mixed venous pO_2 values seen with SFH and SFH-P illustrates the contribution of this variable to the regulation of oxygen transport. Since most reports have considered the mixed venous pO_2 to be a constant value, this observation has rarely been discussed in the literature. The venous oxygen tension thus becomes an additional determinant of oxygen transport equal in significance to cardiac output, hemoglobin mass, or oxygen affinity. The results not only demonstrate the physiologic role of the mixed venous pO_2, but question the importance of different venous pO_2 values as long as the minimum critical oxygen tension is maintained. Furthermore, the concept of a changing venous pO_2 is helpful in understanding the functional

significance of an altered oxygen dissociation curve.

The different venous oxygen tensions observed with SFH and SFH-P are consistent with the data of Riggs.[9] Following exchange transfusion in anemic monkeys with high affinity blood, no changes were seen in cardiac output or arteriovenous oxygen difference. The only difference was a significant fall in mixed venous pO_2, the expected response since all other factors were identical. Unlike the data in our experiment, however, hemoglobin concentrations were unchanged throughout the exchange. Although the mean whole blood and plasma hemoglobin levels were not statistically different in our SFH and SFH-P groups, there was some variation within each group. Further investigation is necessary to clarify the exact relationship between alterations in P_{50} and hemoglobin concentration. It is possible that relatively small differences in hemoglobin concentration might contribute to the effects observed with large changes in P_{50}.

The final issue concerns a difference in physiology between hemoglobin in solution and that present in the red blood cell. The literature includes many experimental and clinical efforts to therapeutically manipulate the red cell hemoglobin oxygen dissociation curve.[10-13] The results show considerable variation. The entire concept of the importance of oxygen affinity and P_{50} is currently being debated, and has recently been reviewed in detail.[14] It is becoming evident, however, that oxygen transport by hemoglobin solution is quite different from that of erythrocytic hemoglobin. The response to acute normovolemic anemia produced by exchange transfusion with Dextran is a linear increase in cardiac output.[1] In contrast, following

exchange transfusion with hemoglobin solution, cardiac output does not change. There is thus a fundamental difference between the two situations. The significance of this altered cardiac response is unclear. It may represent an inability to increase cardiac output, or indicate a more efficient mechanism for maintaining oxygen consumption during normovolemic anemia. A major focus of much of the work on manipulation of oxygen affinity has been an attempt to reduce the effort required by the heart to preserve oxygen transport in situations of increased demand. Since increased cardiac output is not seen even with high oxygen affinity hemoglobin, the oxygen dissociation curve might have a totally different role with hemoglobin solution, with other factors becoming increasingly important. Further work is necessary to explore this possibility.

SUMMARY:

Oxygen dynamics were evaluated in eight baboons exchange transfused with SFH or SFH-P. Oxygen consumption and circulatory dynamics were maintained with both hemoglobin solutions. The oxygen extraction ratio increased in each group, with no change in cardiac output or arteriovenous oxygen content difference. The major finding was the significantly higher mixed venous pO_2 values observed with pyridoxalated hemoglobin at hematocrits below 20. The data suggests that SFH-P is a more physiologically desirable solution than SFH. Finally, the physiology of oxygen transport with hemoglobin solution is different from that of erythrocytic hemoglobin, and may offer cardiac protection during periods of increased demand.

FIGURE 1a

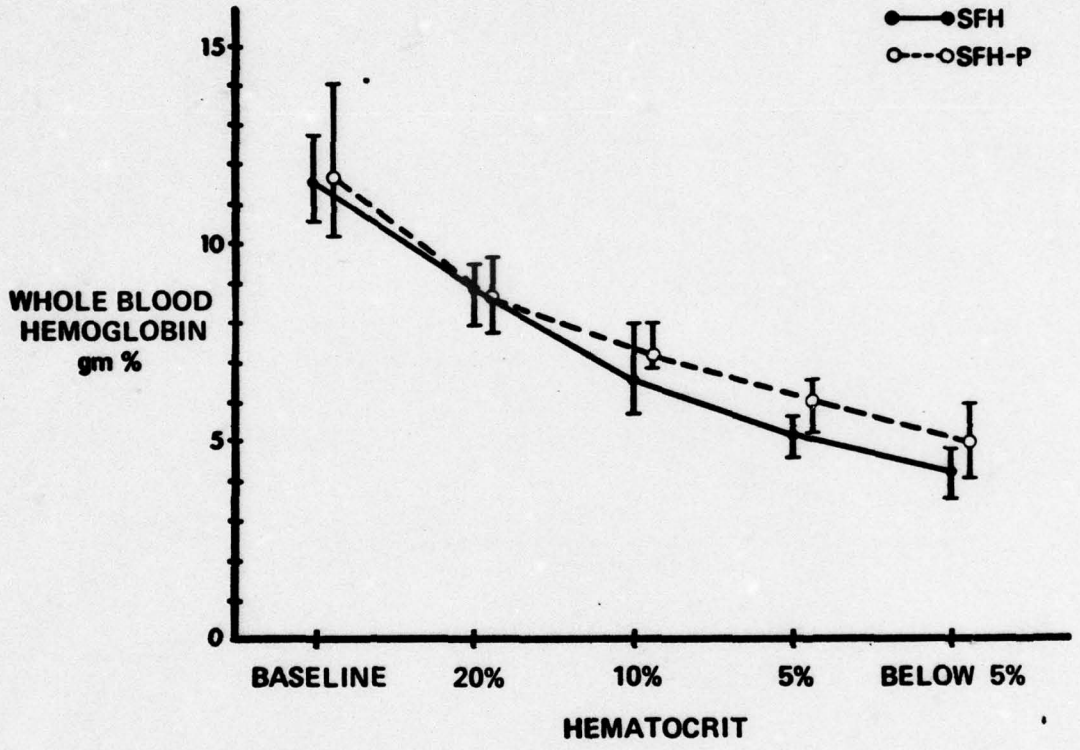

SFH
SFH-P
15
10
5
0
WHOLE BLOOD
HEMOGLOBIN
gm %
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 1b

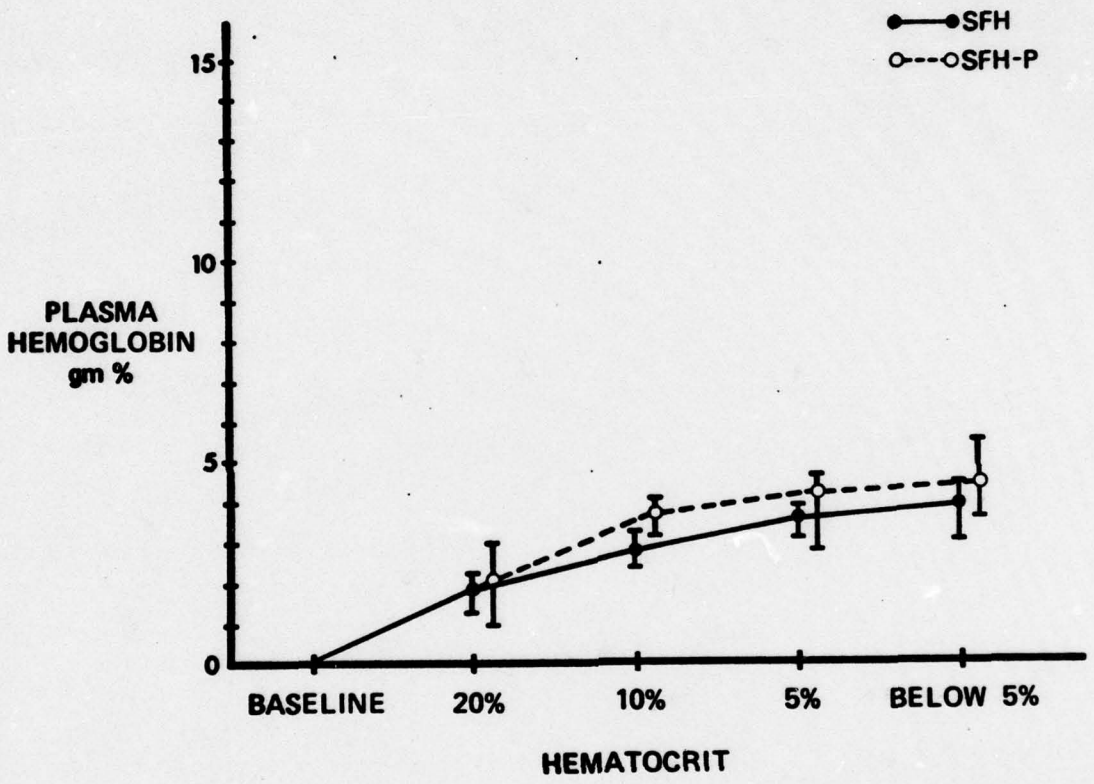

SFH
SFH-P
15
10
5
0
PLASMA
HEMOGLOBIN
gm %
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 2a

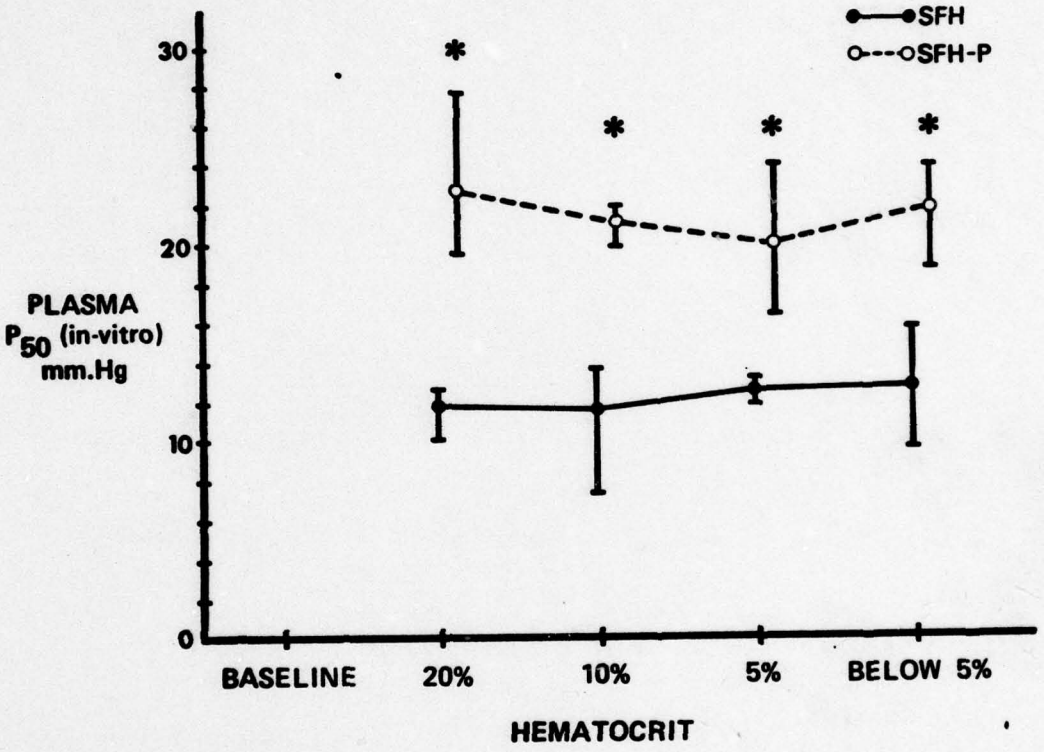

SFH
SFH-P
30
20
10
0
PLASMA
P_{50} (in-vitro)
mm.Hg
*
*
*
*
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 2b

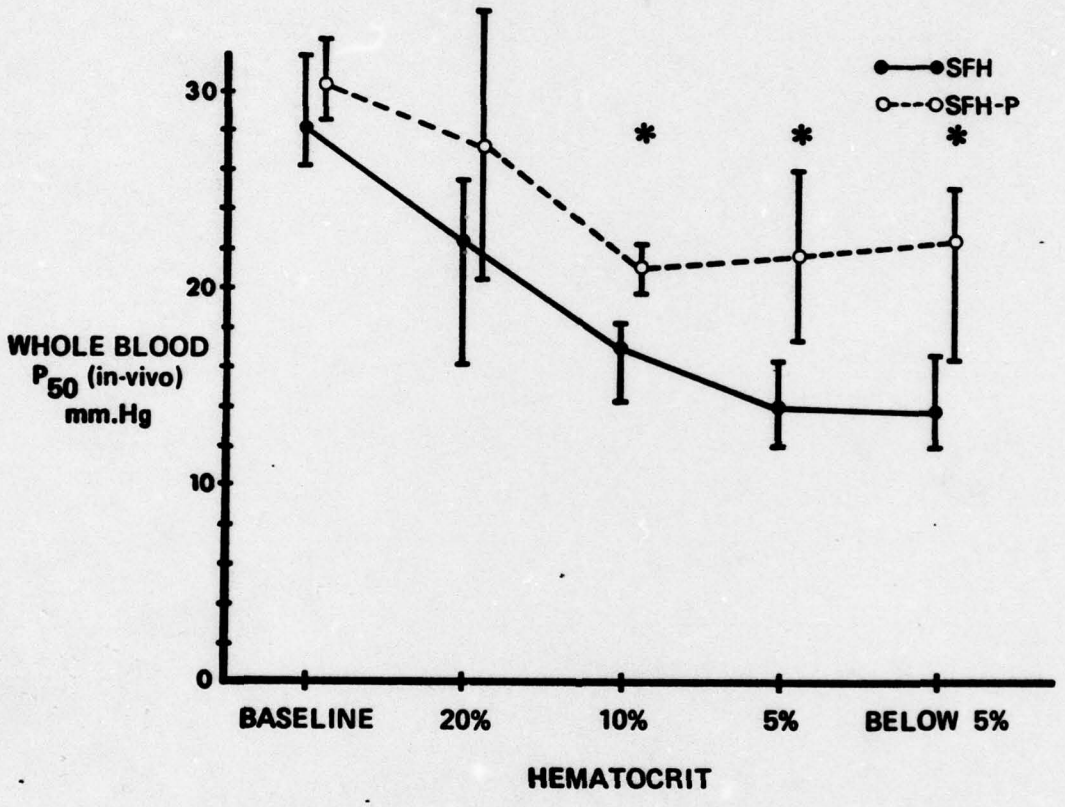

SFH
SFH-P
30
20
10
0
WHOLE BLOOD
P_{50} (in-vivo)
mm.Hg
*
*
*
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 3

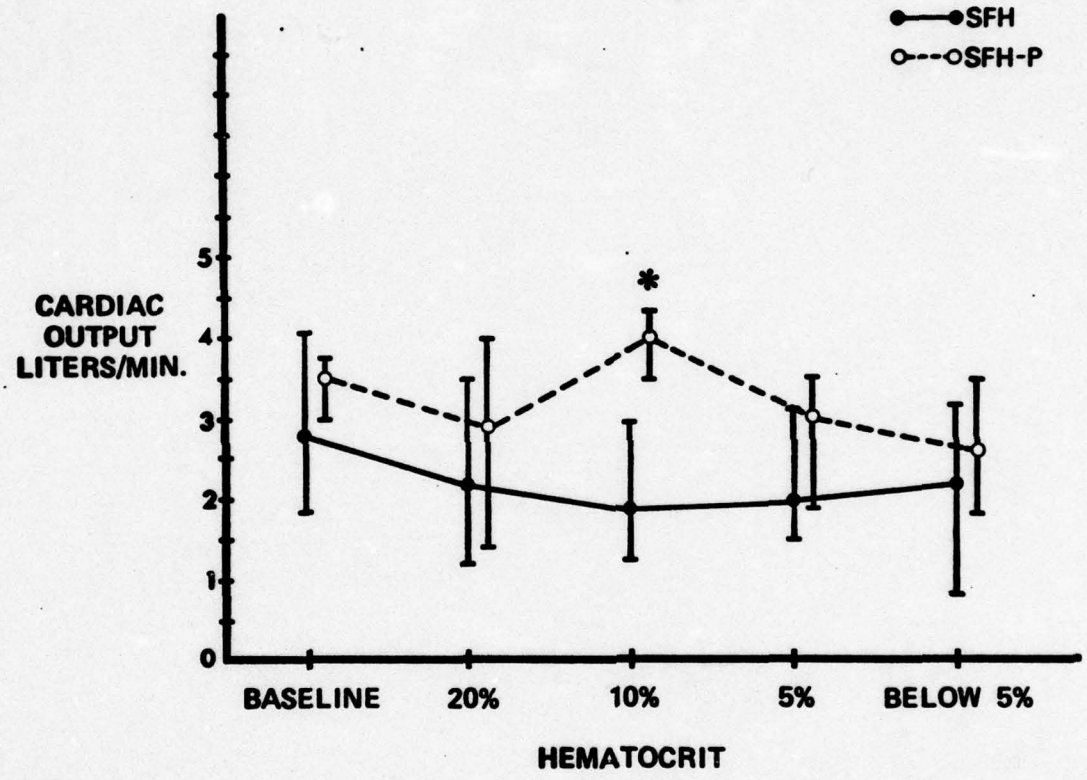

SFH
SFH-P
CARDIAC OUTPUT LITERS/MIN.
0
1
2
3
4
5
*
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 4a

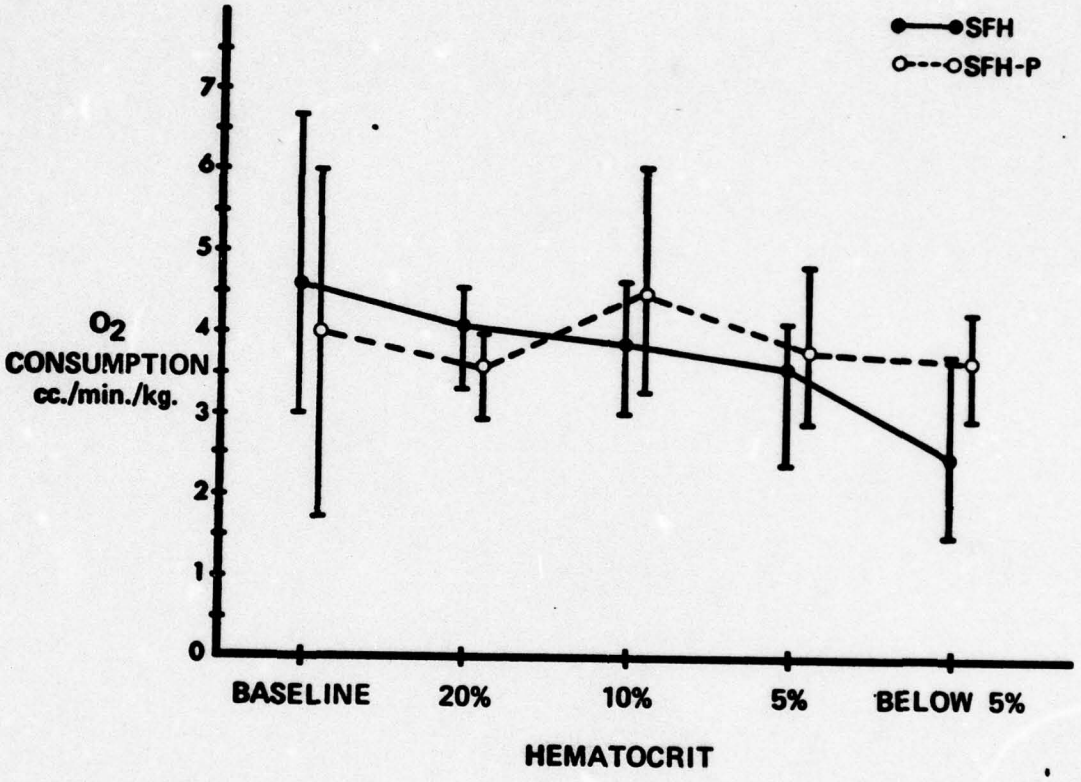

SFH
SFH-P
O_2 CONSUMPTION cc./min./kg.
0
1
2
3
4
5
6
7
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 4b

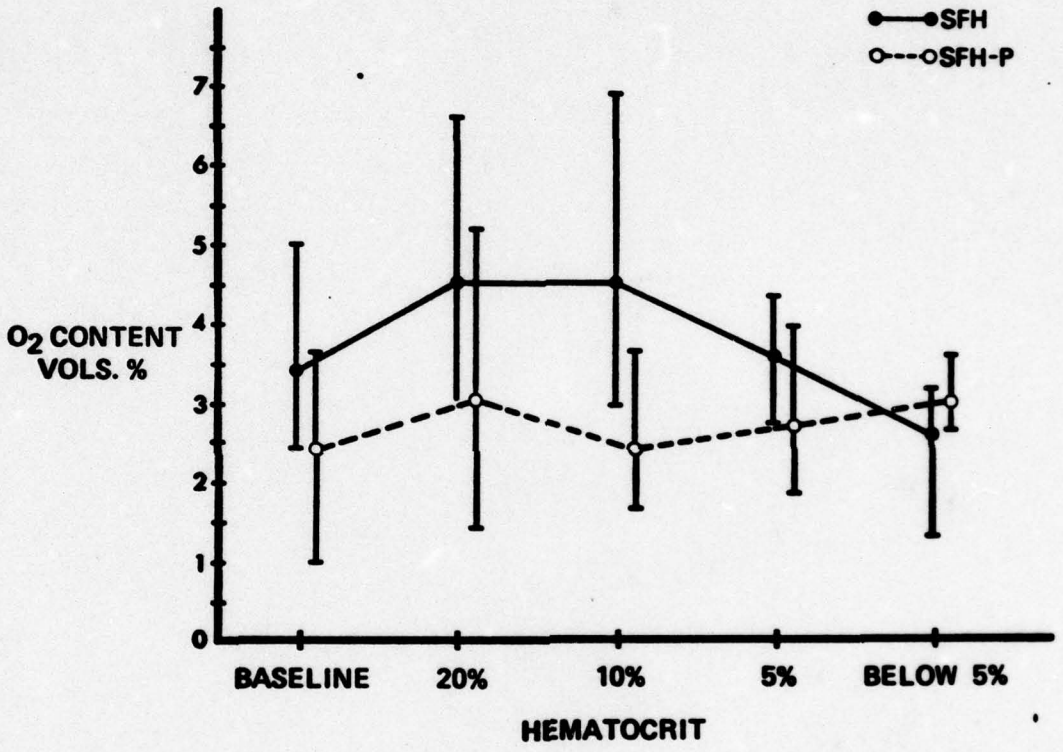

SFH
SFH-P
O_2 CONTENT VOLS. %
0
1
2
3
4
5
6
7
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 4c

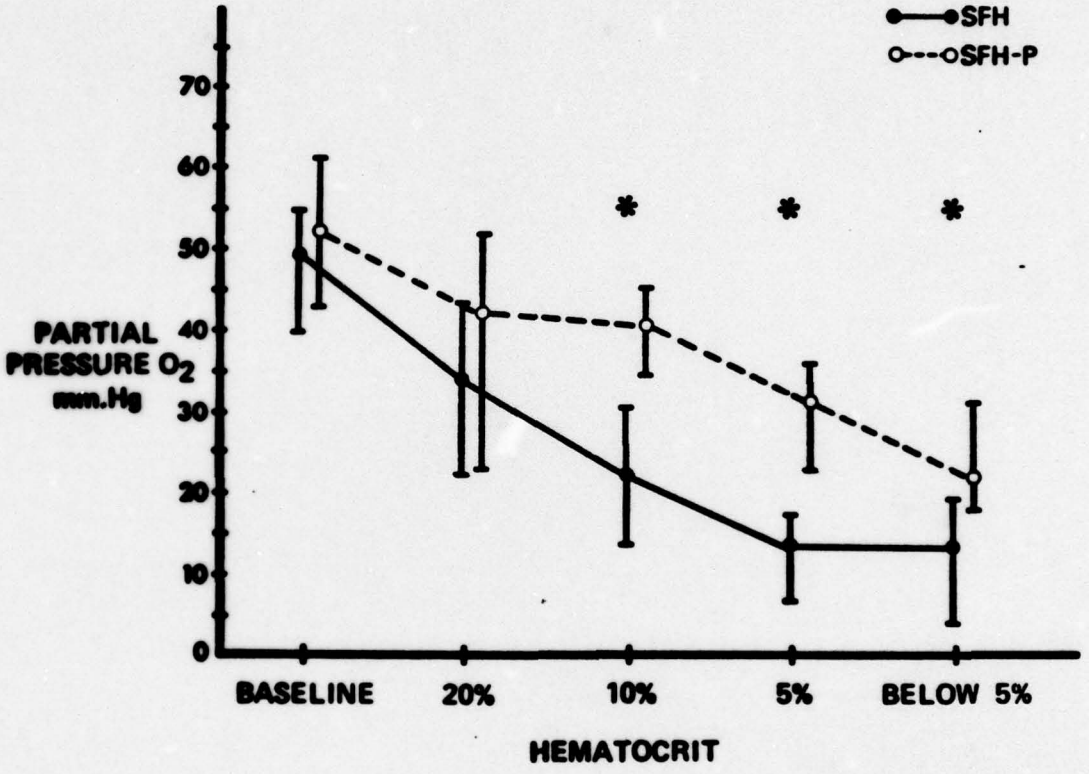
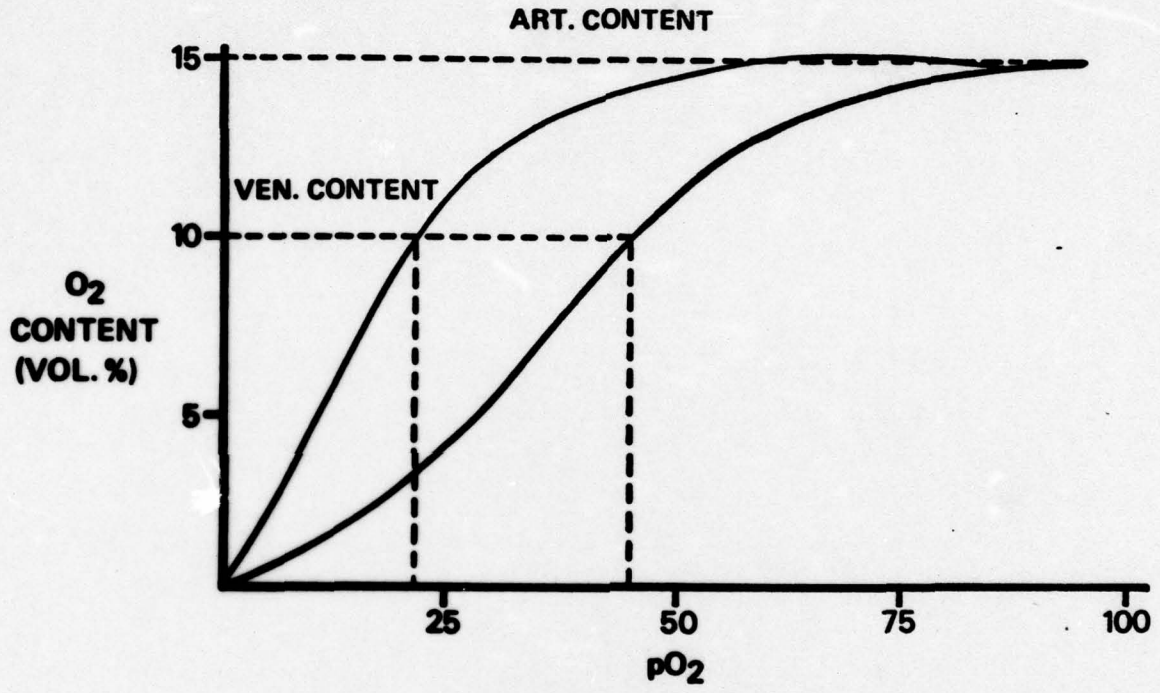

SFH
SFH-P
70
60
50
40
30
20
10
0
PARTIAL PRESSURE O_2 mm.Hg
*
*
*
BASELINE
20%
10%
5%
BELOW 5%
HEMATOCRIT

FIGURE 5



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B. ON-GOING STUDIES

1. CEREBRAL RESPONSE TO RED CELL INFUSION AFTER HEMOGLOBIN ADMINISTRATION

Cerebral metabolism and function can be maintained during moderate normovolemic anemia. The primary compensation appears to be an increase in cerebral blood flow. At hematocrits below about 10%, siginficant alterations occur in the monitored parameters.

Several groups of investigators (1,2,3,4) have reported that after hypotension, ischemia, or severe normovolemic anemia, transfusion of red cells did not reverse defects in observed function or metabolism. We have observed that re-infusion of shed red cells, after the baboon has been at 0 hematocrit and mainted on SFH for several hours, results in a significant flattening of the EEG. On autopsy, the brains appear ischemic.

The purpose of this study will be to study the effect of SFH on cerebral metabolism during normovolemic anemia and subsequent transfusion of shed red cells.

A group of four baboons will be studied. The protocol consists of three stages. Stage 1 is a control period. During Stage 2, the animal is exchanged transfused with SFH to some fixed hematocrit and maintained for three hours. In Stage 3, the washed packed shed red cells are transfused, using physiologic pressures. At the end of Stage 3, the animal is sacrificed and the brain removed for examination.

Measurements will be obtained for each stage. These include the standard hemodynamic, blood gas, and content determinations. In addition, the internal jugular vein will be catheterized to obtain mixed venous samples of cerebral blood. Cerebral blood flow will be estimated using the nitrous oxide technique. Oxygen and glucose concentrations will be obtained for cerebral arterial and mixed venous samples. This data will permit us to calculate oxygen and glucose consumption at each stage of the study. A randomized sequence will be employed to assign the Stage 2 hematocrit level for each study. Final hematocrit levels of 20, 10, 5 and 2 will be used.

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2. RENAL EFFECTS OF STROMA-FREE HEMOGLOBIN SOLUTION

METHODS AND MATERIALS:

Four adult baboons will be the test animals. One week prior to the study, siliconized heparin-filled polyethylene catheters will be surgically implanted into the left renal vein, and the free end ligated and buried subcutaneously. After convalescence from surgery, the renal vein catheter will be re-exposed. Polyethylene catheters will be introduced into the aortic arch and suprarenal vena cava via the femoral vessels. A suprapubic cytotomy will provide urinary drainage. The animals will be loosely restrained in the prone position and allowed to recover from tranquilization.

During the four hour baseline period, the animals will receive an intravenous infusion of normal saline with 44 mEq/L of $NaHCO_3$ at a rate of 10 cc/kg/hr. This infusion will insure a constant basal urine output. A primary dose of PAH (40 mg/kg) and Inulin (75 mg/kg) will be given. This will be followed by a constant infusion of 0.5% PAH and 1.0% Inulin in saline, at 6 cc/kg/hr for the remainder of the experiment. At 60 and 75 minutes after priming, arterial and renal vein samples and fifteen minute urine collections will be obtained for the course of the study.

Every 30 minutes thereafter blood samples will be taken. Beginning with the first 30 minute period, a 50 cc bolus of hemoglobin solution (6 gms %) will be given every 15 minutes.

This will continue until a volume of stroma-free hemoglobin solution (50% of the animals calculated circulating blood volume) has been infused. At the end of this period the animals will be sacrificed and autopsied.

Renal Clearance Determinations: Plasma and urine concentrations of PAH and Inulin will be determined by spectophotometric techniques. The glomerular filtration rate will be determined by:

$$GFR = \frac{\text{(Urinary Conc. of Inulin) x (Urine Flow)}}{\text{(Plasma Conc. of Inulin)}}$$

The renal blood flow will be determined by:

$$\text{Renal Plasma Flow} = \frac{\text{(Urinary Conc. of PAH) x (Urine Flow)}}{\text{(Renal Art. Conc. of PAH - Renal Vein Conc. of PAH)}}$$

$$\text{Renal Blood Flow} = \frac{\text{Renal Plasma Flow}}{\text{1 - Hematocrit}}$$

The free Water Clearance will be determined by:

$$FWC = \frac{\text{(Urine Osmolarity) x (Urine Volume)}}{\text{(Plasma Osmolarity)}} - \text{Urine Volume}$$

The urine and plasma osmolartities will be determined by freezing point depression.

Renal A-V Shunting: To test for renal A-V shunting, blood will be drawn anaerobically for simultaneous blood gas determinations.

3. THE EFFECT OF VARYING HEMOGLOBIN CONCENTRATIONS ON OXYGEN TRANSPORT BY HEMOGLOBIN SOLUTIONS WITH A HIGH OXYGEN AFFINITY

MATERIALS AND METHODS:

Animals. Four adult male baboons (20-22 kg.) will be the test animals. On the morning of each study, the baboons will be tranquilized with an intramuscular injection of 150 mg. of ketamine and 0.4 mg. of atropine, and then sedated with 12-15 mg/kg of thiopental sodium intravenously. The trachea is intubated, and the baboon paralyzed by frequent intravenous injections of d-tuborcurarine. Plastic catheters are inserted into the aorta and vena cava through femoral cutdowns, and a balloon-tipped catheter is flow-directed into the pulmonary artery. A catheter is inserted into the urinary bladder. The baboon is mechanically ventilated with room air in the prone position. In the baseline period the rate and tidal volume are adjusted to produce an arterial pCO_2 between 35 and 45 millimeters of mercury, and these ventilator settings are held constant throughout the study.

Exchange Transfusion. Following stabilization of the animal and baseline measurements, blood will be withdrawn in 50 milliliter aliquots from an arterial catheter and simultaneously replaced with an equal volume of hemoglobin solution (12 gms%) infused into the venous catheter. The exchange will be interrupted at hematocrits of 20, 10, 5, 2, and zero for a complete series of measurements. The exchange will continue until the lowest hematocrit is achieved. When zero hematocrit is reached, the animals will be diluted using Lactated Ringer's from a hemoglobin of 12 gms% to 2 gms %. The silution will be interrupted for measurements at 10, 8, 6, and 4 gms.%.

Measurements. All samples are collected and separated anaerobically in order to perform analyses of both the plasma and erythrocytic hemoglobin compartments. Hemoglobin and methemoglobin levels, as well as arterial and venous oxygen contents and saturations are measured in a cooximeter (Instrumentation Laboratory, Model 282). Arterial and venous blood gases and pH are measured using standard electrodes. Oxygen dissociation curves are generated on a Hem-O-Scan (American Instrument Co.) at a constant temperature (37°C) and pCO_2 (40 mm. Hg.). Standard corrections for pH, temperature, and pCO_2 yield in-vitro (pH=7.4, pCO_2=40, temp =37°C) and in-vivo (animal conditions) P_{50} values.

Cardiac output is determined by the thermodilution technique. Arterial pressure, CVP, PAP, and PAW are measured from the aortic and pulmonary artery catheters.

Oxygen consumption is calculated as the product of the cardiac output and arteriovenous oxygen content difference. Oxygen availability is calculated as the product of the cardiac output and arterial oxygen content, and the oxygen extraction ratio is determined as the ratio of consumed to available oxygen.

Hemoglobin Preparation. SFH is prepared from outdated human red cells by a modification of our previous technique[1], yielding a final product with a P_{50} of 12 to 14 mm. Hg. and a Hgh conc. of 12 gms %.

Statistics. Results will be represented as a regression of hemoglobin concentration versus arterial and venous gases, cardiac output, etc.

Results. This study will allow us to determine the effect of increasing or decreasing hemoglobin concentration as compared to the previously studied effects of increasing or decreasing the P_{50}, and therefore determine the relative value of either manipulation.

C. BUDGET AND BUDGET JUSTIFICATION

BUDGET

1.	**SALARIES**	
	A. Biochemist	$ 18,360.00
	Fringe Benefits	1,126.00
	Overhead, 75% of S+W	13,770.00
	B. Biochemistry Technician	$ 14,040.00
	Fringe Benefits	861.00
	Overhead, 75% of S+W	10,530.00
	TOTAL	$ 58,687.00
2.	**SUPPLIES**	
	A. Baboons - 12 @ $648.00	$ 7,776.00
	B. Animal Care, Approx. $120.00/mo	1,440.00
	C. Surgical Charges	
	$90/surgery x 12 baboons	1,080.00
	D. Expendable Supplies	700.00
	GRAND TOTAL	$ 69,683.00

BUDGET JUSTIFICATION

PERSONNEL: The salary levels are consistent with the prevailing job market in Chicago. Two people are necessary because of the nature of the studies plus the fact that we will be producing large batches of stroma free hemoglobin solution for designated investigators as well as studying its properties ourselves.

BABOONS: Four (4) for the varying Hgb-concentration study, four (4) for the Renal Study, and four (4) for the Cerebral Response Study. Twelve (12) baboons at $648.00 each equals a total of $7,776.00. The average monthly charge for animal care is $120.00 for twelve (12) months, which equals $1,440.00 per year.

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